

Podocyte expression of the CDK-inhibitor p57 during development and disease

KEIJU HIROMURA,¹ LEAH A. HASELEY,¹ PUMIN ZHANG, TOSHIAKI MONKAWA, RAGHU DURVASULA, ARNDT T. PETERMANN, CHARLES E. ALPERS, PETER MUNDEL, and STUART J. SHANKLAND

Department of Medicine, Division of Nephrology, University of Washington School of Medicine, Seattle, Washington; Program in Cell Biology, National Jewish Medical and Research Center, Denver, Colorado; Department of Pathology, University of Washington School of Medicine, Seattle, Washington; and Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York, USA

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Background. The mature podocyte is a terminally differentiated cell with a limited proliferative capacity. The precise cell cycle proteins necessary for establishing podocyte quiescence during development or permitting podocyte cell cycle re-entry in disease states have not been fully defined. Accordingly, we studied the role of the cyclin dependent kinase (CDK)-inhibitor p57^{Kip2} (p57) in modulating these processes.

Methods. The expression of p57 protein in relation to markers of DNA synthesis was examined in developing mouse kidneys, and in the passive Heymann nephritis (PHN) and anti-glomerular antibody models of glomerular disease by immunohistochemistry. The role of p57 in glomerulogenesis was explored by examining renal tissue from embryonic p57^{-/-} mice, and the expression of p21, p27 and p57 protein and mRNA was examined in podocytes in vitro.

Results. The de novo expression of p57 during glomerulogenesis coincides with the cessation of podocyte proliferation, and the establishment of a mature phenotype, and p57 is expressed exclusively in podocytes in mature glomeruli. However, p57 knockout mice have normal glomerular podocyte development. In addition, mRNA but not protein levels of p57 increased upon differentiation of podocytes in vitro. There was a marked decrease in p57 expression in both animal models of podocyte injury. This was diffuse in PHN, whereas in the murine model, loss of expression of p57 occurred predominantly in proliferating podocytes, expressing proliferating cell nuclear antigen (PCNA).

Conclusion. Despite the de novo expression of p57 protein coinciding with the cessation of primitive podocyte proliferation during glomerulogenesis, embryonic p57^{-/-} mice glomeruli were histologically normal. Cultured podocytes did not re-

quire changes in p57 protein levels to undergo differentiation. These data suggest that p57 alone is not required for podocyte differentiation, and that other cell cycle regulators may play a role. Furthermore, although injury to mature podocytes in experimental glomerular disease is associated with a decrease in p57, the levels of all three members of the Cip/Kip family of CDK inhibitors appear to determine the capability of podocytes to proliferate.

Podocyte injury is the primary event in the initiation of a diversity of glomerular diseases including those caused by immune, metabolic, toxic, and hemodynamic events [1]. Whereas the injury response of the mesangial or endothelial cell often includes cell proliferation, the injured podocyte, or glomerular visceral epithelial cell, differs as this cell type has a very limited capacity for cell division [2–4]. Studies in both animal models and human disease suggest that while the podocyte is capable of DNA synthesis, the replicative process may stall after mitosis resulting in nuclear polyploidy and cell hypertrophy without an increase in podocyte number [5, 6]. Evidence suggests that this failure to divide may account for the relentless process of glomerulosclerosis leading to irreversible renal failure [7].

Primordial and immature podocytes freely enter the cell cycle and proliferate during embryogenesis [8]. Mature podocytes then exit the cell cycle in order to take on a terminally differentiated phenotype [9]. Mature podocytes, however, may re-engage the cell cycle in certain disease states [10]. Yet the factors establishing and maintaining podocyte quiescence in development, and those permitting cell cycle re-entry of diseased mature podocytes are poorly understood. Cell proliferation is strictly governed at the level of the cell cycle [11]. Following mitogenic stimuli, cells exit quiescence (G₀) and transit through G₁ into the S phase, where DNA synthesis occurs

¹Drs. Hiromura and Haseley contributed equally to this article.

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[12]. Thereafter, cells pass through G_2 into the final M (mitotic) phase, followed by cytokinesis. Recently it has been appreciated that the cell cycle is a tightly regulated process dependent on both positive and negative regulatory influences [13, 14]. Cyclins and their catalytic subunits cyclin dependent kinases (CDKs) constitute the major positive regulatory proteins [15]. During each phase of the cell cycle, there is increased expression of specific cyclins. Thus, D-type cyclins are expressed early in G_1 , cyclin E in late G_1 , cyclin A in G_1/S , and cyclin B in the M phase. Cyclins bind to and activate specific CDKs. D-cyclins bind to CDK4, whereas cyclins E and A bind to CDK2. Cyclin B binds to cdc2 (formally called CDK1). In turn, active CDKs phosphorylate certain target genes required for DNA synthesis, such as pRB [15]. The cell cycle also is negatively regulated by a group of proteins known as cyclin kinase inhibitors (CKI) [14]. CKI cause cell cycle arrest by binding to and inhibiting specific cyclin-CDK complexes. The Cip/Kip family of CKI, consisting of p21^{CIP1}, WAF1, SDI1 (p21), p27^{KIP1} (p27), and p57^{KIP2} (p57), which inhibit both G_1 and S phase cyclin-CDK complexes, have been best studied with regard to their role in glomerular disease [16, 17]. In particular, we have shown that the dramatic mesangioproliferative response following complement-mediated injury in the Thy1 model is associated with down-regulated expression of p27 [18]. In contrast, in the passive Heymann nephritis model of membranous nephropathy, injury to the podocyte is associated with up-regulation of the CKI p21 and p27 [19]. Studies of nephritic p21 and p27 knockout mice have further supported a role for these CKI in limiting podocyte proliferation [20, 21].

The most recently identified member among the Cip/Kip family of CKIs is p57. Named according to its molecular weight, p57 is a 57 kD protein that inhibits cell cycle progression by binding to CDK2, -3, and -4 [22, 23]. Overexpressing p57 leads to G_1 phase cell cycle arrest [22, 23]. Mouse p57 exhibits unique protein domains (proline-rich and acidic) that are structurally distinct from those common to p21 and p27 [23]. In contrast to the widespread tissue expression of p21 [24] and p27 [25], p57 demonstrates a more restrictive distribution in placenta, muscle, heart, brain, lung and kidney [22, 23]. p57 has been implicated in the process of cell cycle exit accompanying terminal differentiation of a variety of non-renal cells including lens fibers [26, 27], myoblasts [28], and keratinocytes [29].

We [30] and others [31] have previously reported that p57 is constitutively expressed in mature podocytes. Yet the role of this CKI in the regulation of podocyte maturation and proliferation, and in glomerular disease has not been clearly defined. Accordingly, to explore the possibility that p57 is a critical regulator of podocyte proliferation and differentiation during development and in disease, we examined the protein expression of p57 during

murine nephrogenesis, in cell culture, and in experimental models of glomerular disease. Our results show that although p57 expression coincides with podocyte exit from the cell cycle during glomerulogenesis, p57^{-/-} mice have a normal glomerular architecture. We also show that the mRNA levels for p21 and p27, p57 increased in cultured podocytes during differentiation. Finally, our results show that injury to podocytes in vivo is associated with a decrease in p57 expression, which alone is not sufficient to induce podocyte proliferation.

METHODS

In vivo studies

Kidney development. To determine the temporal expression of p57 during podocyte development, embryonic kidneys of gestational ages embryonic day 15 (E15; $N = 5$), E18 ($N = 5$), and E21 ($N = 4$) were harvested from gravid C57BL6 mice (Simonsen, Gilroy, CA, USA). Additional kidneys were removed from mice sacrificed at days 1 ($N = 10$), 3 ($N = 9$), 5 ($N = 9$), 7 ($N = 8$), 14 ($N = 8$), and 28 ($N = 8$) following birth. In a separate experiment, two gravid mice of gestational age E15 were injected with intraperitoneal 5-bromo-2'-deoxyuridine (BrdU, 10 μ L/g; Amersham Life Science, Arlington Heights, IL, USA) four hours prior to sacrifice. All kidneys were fixed in methyl Carnoy's solution for immunohistochemistry.

To determine the role of p57 during glomerulogenesis, p57 heterozygotes (on a C57BL6 background) were bred to generate p57 null (p57^{-/-}) and wild-type (p57^{+/+}) offspring as previously reported [32]. Heterozygote gestational female mice were injected with 10 μ L/g BrdU (Amersham) four hours prior to sacrifice. Embryonic kidneys were removed on days E15 and E18 and fixed in methyl Carnoy's solution to study glomerular morphology and cellularity in p57 null and wild-type mice ($N = 3$ per time point/group), which were identified by standard genotyping. Attempts were made to study postnatal glomerulogenesis, but no p57 null mice survived to birth [33].

Experimental model of podocyte injury in the rat. To examine the expression of p57 in vivo following podocyte injury that is characterized by an absence of podocyte proliferation, the passive Heymann nephritis (PHN) model of membranous nephropathy (a model devoid of podocyte proliferation) was studied. PHN was induced in male Sprague-Dawley rats (180–200 g; Simonsen) by the intraperitoneal injection (5 mL/kg) of sheep antibody to Fx1A [34]. A control group consisted of rats receiving normal sheep serum. Control and PHN animals were sacrificed on days 5 ($N = 6$ per group), 10 ($N = 5$ per group) and 30 ($N = 6$ per group) following disease induction.

Experimental model of podocyte proliferation in the mouse. In order to correlate p57 expression with podocyte

cyte proliferation in vivo, immune-mediated glomerular injury was induced in C57/BL6 mice (Simonsen) by the intraperitoneal injection of sheep anti-rabbit glomerular antibody (0.5 mL/20 g body weight) on two consecutive days, as previously described [20, 34]. This model is characterized by low-grade glomerular epithelial cell proliferation [20, 34]. Mice receiving normal sheep serum served as controls. Kidneys were harvested on day 7 following disease induction ($N = 6$ per group).

Immunohistochemistry. Kidneys from rats or mice were fixed in methyl Carnoy's solution, processed and embedded in paraffin using conventional techniques. Two-micrometer thin sections were stained for p57 using the avidin/biotin indirect immunoperoxidase method as previously described [18]. In brief, sections were incubated overnight with the primary antibody M20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a goat polyclonal IgG that recognizes an epitope mapping to the amino terminus of mouse p57. The specificity of the p57 antibody was verified by Western blotting wherein the primary antibody was mixed with serial dilutions of a p57 blocking peptide (Santa Cruz). This was followed by a secondary biotinylated rabbit antigoat IgG (Zymed, San Francisco, CA, USA) and horseradish peroxidase streptavidin (Vector, Burlingame, CA, USA). To determine if p57 immunostaining colocalized with DNA synthesis, sequential sections also were stained for the DNA synthesis markers proliferating cell nuclear antigen (PCNA) and BrdU. For PCNA staining, we used the primary antibody 19A2, a murine IgM (Coulter, Hialeah, FL, USA), followed by an HRP-Rat antimouse IgM (Zymed); for BrdU staining, the primary and secondary antibodies contained within the Amersham cell proliferation kit (Amersham) were used in conjunction with a microwave retrieval step, as previously described [36]. Diaminobenzidine (Sigma Chemicals, St. Louis, MO, USA) with (for PCNA and BrdU immunostaining) or without (for p57 immunostaining) nickel chloride was used as the detecting reagent. Negative controls consisted of omission of the primary antibody, or substitution of the primary antibody with an irrelevant murine immunoglobulin.

Cell culture studies

Conditionally immortalized mouse podocytes in cell culture: Experimental design. We used a conditionally immortalized mouse podocyte cell line for the cell culture experiments, and have referred to these cells as heat-sensitive mouse podocytes (HSMP) in this article. The methods for producing H-2K^b-tsA58 transgenic mice [36], and isolating podocytes from H-2K^b-tsA58 kidneys have been described previously [37]. These mice are transgenic for a temperature-sensitive (tsA58) SV40 large T antigen that is under control of a γ -interferon inducible promoter. When podocytes derived from these mice are cultured under growth permissive conditions

(33°C and media supplemented with mouse γ -interferon 50 units/mL), they readily proliferate and maintain a uniform, cobblestone morphology (referred to as "growth permissive" in this article). In contrast, podocytes grown under differentiating conditions (37°C, no interferon) stop proliferating, and elaborate a complex network of processes. This transformation to an "arborized" phenotype (referred to as the "differentiating" phenotype) is associated with a marked diminution in DNA synthesis, and the de novo expression of synaptopodin, a marker of mature podocytes [38].

Growth permissive and differentiating HSMP were grown on plastic in RPMI (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS). Recombinant mouse γ -interferon (Coulter) was added only to growth permissive media at a concentration of 50 U/mL. Cells from passages 11 to 19 were used for all experiments, which were performed a total of four times. Except where noted, studies were performed on cells at day 3 following passage in growth permissive conditions, and at days 3 and 8 following passage into differentiating conditions.

BrdU, WT-1, and synaptopodin staining in vitro. 5-Bromo-2'-deoxyuridine incorporation into DNA was used as a measure of DNA synthesis. Cells were passaged and grown in chamber slides (50,000 cells/well) under growth permissive and differentiating conditions. On sequential days following passage (days 2 to 9), cells were incubated for four hours in serum free media containing BrdU (Amersham) at a concentration of 1:500. Cells were then fixed in a 1:1 mixture of methanol and acetone for 15 minutes at -20°C. Immunofluorescent staining for BrdU was carried out using the nuclease and primary antibody contained in the Amersham cell proliferation kit (Amersham) with the exception that the primary antibody was used at double the concentration recommended by the manufacturer. This was followed by a biotinylated antimouse IgG (Vector) and streptavidin FITC (Amersham). Counterstain was with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) at a concentration of 10 μ mol/L. The number of BrdU positive cells per 300 Hoechst positive nuclei was recorded for each time point.

For WT-1 and synaptopodin staining, cells were grown in chamber slides under both permissive (day 3) and differentiating (day 6) conditions, and were fixed in methanol and acetone as described earlier in this article. For WT-1 staining, the primary rabbit polyclonal IgG antibody (C-19; Santa Cruz Biotechnology) was incubated overnight at 4°C. This was followed by a biotinylated anti-rabbit IgG (Vector), and streptavidin FITC (Amersham). For synaptopodin staining, the primary antibody (generated by Dr. Peter Mundel as previously described) consisted of a murine monoclonal anti-synaptopodin IgG. This was incubated overnight, followed by a biotinylated antimouse IgG (Vector) and streptavidin FITC (Amer-

sham). Cells were counterstained with Hoechst 33342 (Molecular Probes).

Measuring the expression of cell cycle regulatory proteins in cultured cells

Quantitative real-time PCR for p21, p27 and p57. The mRNA levels of the CDK-inhibitors p21, p27 and p57 were measured by real-time polymerase chain reaction (PCR) and quantitated by measuring the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA was isolated from 70% confluent HSMP using the Tri-Reagent™ (Sigma) according to manufacturer's instructions. Five micrograms of total RNA were converted into cDNA using the oligo(dT) protocol contained in the Superscript™ First-Strand Synthesis System for reverse transcription (RT)-PCR (Gibco BRL). Twenty-five nanoliters of the RT reaction (20 µL) were then used as template for a 50 µL PCR reaction (1× TaqMan Universal Master Mix; Applied Biosystems, Foster City, CA, USA), 200 nmol/L forward and reverse primers (100 nmol/L TaqMan probe) for p21, p27, p57 and GAPDH using the ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems). Primers and TaqMan probes were designed by the Primer Express™ 1.0 (Applied Biosystems) as follows: p21 [forward primer, 5'-CCGTTGTCTCTT CGGTCCC-3', reverse primer, 5'-CATGAGCGCATC GCAATC-3', a TaqMan probe, 5'-(FAM)-TGGACAG TGAGCAGTTGCGCCG-(TAMRA)-3'], p27 [forward primer, 5'-TTTTCCGGAGAGAGGCGAG-3', reverse primer, 5'-CTCACGTTTGACATCTTCCTCT-3', TaqMan probe, 5'-(FAM)-CGGTGGTCCACACCCGCCC-(TAMRA)-3'], and p57 [forward primer, 5'-CAGCGGACGATGGAAGAACT-3', reverse primer, 5'-CTCCGGTTCCTGCTACATGAA-3', TaqMan probe, 5'-(FAM)-TGGGCTTCGGCTGGGACCTTTC-(TAMRA)-3']. TaqMan probes for p21, p27 and p57 were purchased from Synthesgen (Houston, TX, USA). Primers and TaqMan probe for GAPDH were obtained from TaqMan rodent GAPDH control reagent (Applied Biosystems).

The PCR conditions were: two minutes at 50°C, ten minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for one minute. As a standard, a stock cDNA made from RNA obtained from differentiating HSMP was serially diluted in tenfold steps in water from 250 nL to 250 pL. The standard curve was calculated based on the threshold cycles (Ct) values using Sequence Detector™ 1.7 (Applied Biosystems) and relative mRNA quantity in each sample was determined based on its Ct value. To normalize the quality and quantity of mRNA in each sample, p21, p27 and p57 mRNA levels were estimated as the ratio of p21/GAPDH, p27/GAPDH and p57/GAPDH. The results obtained from growth restrictive conditions are reported as the magnitude of increase relative to growth permissive conditions.

Western blot analysis

The protein expression of certain cell cycle regulatory proteins was measured by Western blot analysis as previously described [38]. Confluent HSMP were washed three times with phosphate-buffered saline (PBS), and detached using a cell scraper. Following centrifugation, pellets were suspended in a buffer containing 1% triton, 10% glycerol, 20 mmol/L HEPES, 100 mmol/L NaCl, and the Complete™ protease inhibitor tablets (Boehringer Mannheim, Indianapolis, IN, USA). Following a vortex, HSMP were stored overnight at -70°C. The protein concentration of the supernatant was measured by the BCA protein assay (Pierce, Rockford, IL, USA). For Western blot analysis, HSMP protein (10 µg for cyclin A, B1, CDK2, p21, p27; 20 µg for cyclin D2; 20 to 100 µg for p57) extract was separated under reducing conditions on either 8% (cyclin A, cyclin B1, p57) or 15% (CDK2, cyclin D2, p21, p27) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) by electroblotting. Non-specific background was blocked by incubating the membranes with 5% non-fat dried milk for 30 minutes. Blots were probed overnight at 4°C using rabbit polyclonal antibodies to cyclin A (Santa Cruz) and cyclin D2 (Santa Cruz), mouse monoclonal antibodies to CDK2, cyclin B1 (Santa Cruz), p21 (PharMingen, San Diego, CA, USA), and p27 (Santa Cruz), or a goat polyclonal antibody to p57 (Santa Cruz). For p57, the secondary antibody consisted of a biotinylated anti-goat IgG (Zymed) followed by streptavidin-alkaline phosphatase (Vector). An alkaline phosphatase conjugated secondary antibody (Promega, Madison, WI, USA) was used in blots for all other cell cycle regulatory proteins. Protein was detected with the chromagen 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Immunoblots were performed in duplicate or triplicate on protein obtained from four to six separate experiments. Controls included omission of the primary or secondary antibodies; another transformed and late passage podocyte cell line was used as a positive control for p57. India ink staining was used to verify equivalent protein loading.

Statistical analysis

Statistical analysis on data obtained for BrdU staining and densitometry was performed using analysis of variance (ANOVA) with a Bonferroni-Dunn correction (Statview; Abacus Concepts, Berkeley, CA, USA). A *P* value <0.05 was considered statistically significant.

RESULTS

p57 was expressed in a specific spatial and temporal pattern during murine nephrogenesis, and is constitutively expressed in mature podocytes

To determine the localization and onset of p57 protein expression in podocytes, we began by examining the kid-

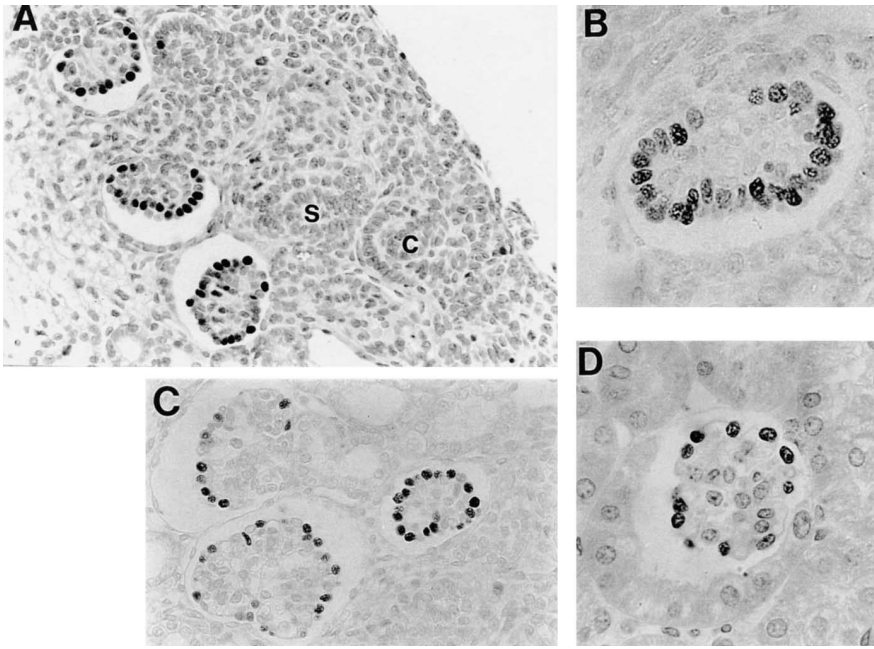


Fig. 1. Indirect immunoperoxidase immunostaining for p57^{Kip2} (p57) during murine glomerulogenesis. (A) The developing murine cortex at E15 displays glomeruli at all stages of maturation ($\times 200$). p57 is absent in early glomerular structures including comma (example marked by C) and most S-shaped bodies (example marked by S). p57 is expressed strongly by cells at the capillary loop and maturational stages, and p57 expression is seen only in cells on the exterior of the capillary loops, consistent with developing podocytes. (B) High power view ($\times 630$) of a glomeruli at the capillary loop stage showing intense nuclear staining for p57. (C) In maturational glomeruli at E18, p57 expression is segmental, a pattern which persists in adulthood ($\times 400$). (D) Glomerulus from a normal adult mouse shows persistent staining for p57 in a podocyte distribution ($\times 1000$).

neys of embryonic mice. Staining for p57 was not detected in the early glomerular structures including vesicles and comma-shaped bodies (Fig. 1A). Among S-shaped bodies from all embryonic time points, $<1\%$ of cells exhibited faint staining for p57. In contrast, virtually all epithelia resembling primordial podocytes demonstrated intense nuclear staining for p57 at the capillary loop stage (Fig. 1A). There was strong nuclear staining for p57 in cells situated on the exterior of the capillary loops, in a distribution consistent with podocytes (Fig. 1B). Segmental, but persistent intense immunostaining was observed in podocytes of mature glomeruli (Fig. 1C).

Immunohistochemical staining was also performed on kidneys from unmanipulated adult mice. Expression of p57 was not detected in mesangial, glomerular endothelial, or parietal epithelial cells (Fig. 1D). Although p57 was not detected in every podocyte, the pattern of immunostaining for p57 was global, and present in the majority of podocytes of every glomerulus. The pattern for p57 immunostaining was similar in podocytes of the adult rat (Fig. 4A).

p57 expression coincided with the cessation of DNA synthesis during glomerulogenesis

To determine the colocalization of p57 and cell proliferation, murine embryonic tissue was stained for BrdU and PCNA, markers of DNA synthesis, on serial sections alongside staining for p57. As shown in Figure 2, the staining pattern for BrdU was essentially reciprocal to that observed for p57. BrdU staining was most intense in the nephrogenic zone of the outer cortex, corresponding to the location of the most primitive glomerular struc-

tures. BrdU staining was markedly diminished in glomeruli at the capillary loop and maturational stages, with virtually no BrdU positive cells detectable in a distribution consistent with podocytes (Fig. 2). A similar pattern of PCNA staining was observed at all embryonic time points and at days 1, 3, and 5 following birth. Thereafter, PCNA staining diminished markedly, with rare PCNA positive cells detectable in the glomeruli of one-week-old mice. In biopsies of 14-day-old mice, PCNA was essentially restricted to renal tubular epithelia (results not shown). These results show that p57 and markers of DNA synthesis do not colocalize during glomerulogenesis, and suggest that p57 may be required for the decrease in podocyte DNA synthesis during development.

p57 alone was not required for normal glomerular maturation

To determine the role for p57 in glomerular development, we examined kidneys from embryonic p57^{-/-} and p57^{+/+} mice. PAS staining on day 15 and day 18 kidneys showed that the histologic pattern of glomerulogenesis was similar in both mice, and that the kidneys from p57^{-/-} mice displayed normal glomeruli at all stages of maturation, including capillary loop and mature forms (Fig. 3). There were no gross defects in podocyte maturation detected on routine light microscopy, and there was no increase in glomerular cellularity in p57^{-/-} mice. To determine if the lack of p57 was associated with an increase in DNA synthesis, gravid mothers were injected with BrdU prior to sacrifice. There were no differences in the pattern or intensity of glomerular immunostaining for either BrdU or PCNA in the p57^{-/-}

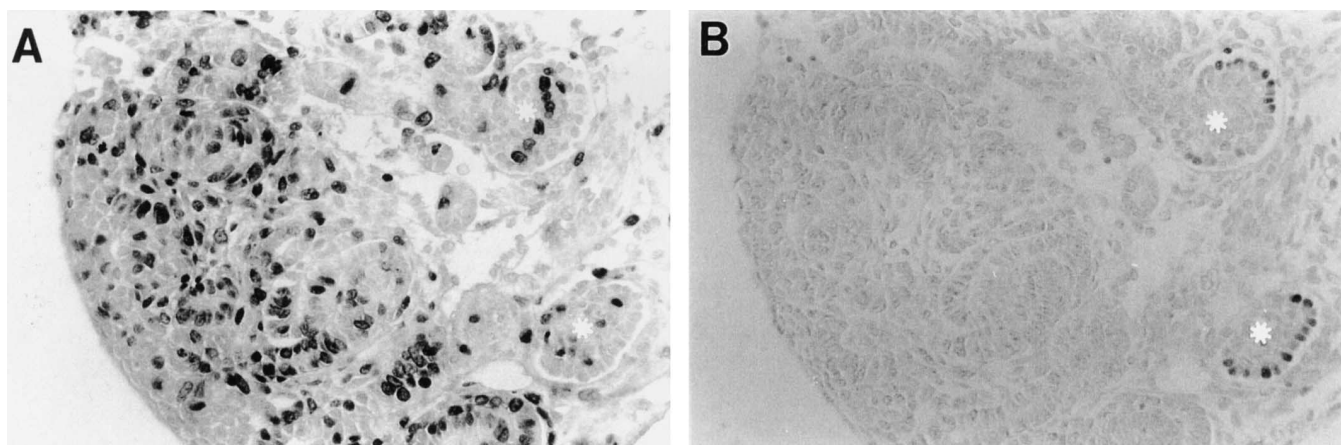


Fig. 2. p57 expression coincides with the cessation of DNA synthesis during murine glomerulogenesis. (A and B) Serial sections from a wild-type mouse embryo at E15. (A) BrdU staining. BrdU, a marker of DNA synthesis, is widely expressed in primitive glomerular structures concentrated in the outer cortex. BrdU staining is not detected in podocytes of glomeruli at the capillary loop and maturational stages (example shown by asterisks). (B) p57 staining. In contrast to BrdU, p57 staining is limited to cells that are BrdU negative (serial glomerulus shown by asterisks), and therefore not undergoing DNA synthesis ($\times 400$). In mature glomeruli, p57 staining was in a typical podocyte distribution.

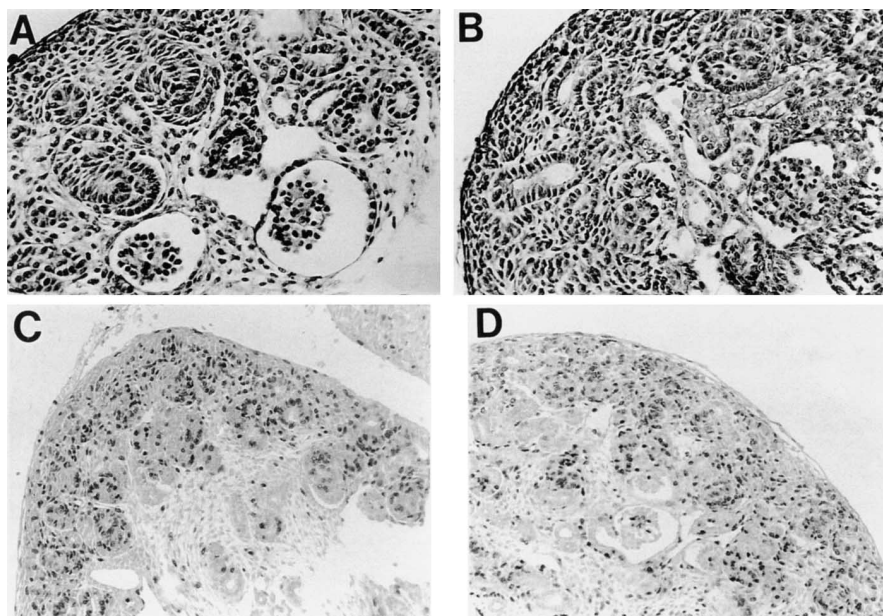


Fig. 3. p57 is not required for normal glomerulogenesis. Serial sections for periodic acid Schiff (PAS) and BrdU staining in p57+/+ (A, C) and p57-/- (B, D) E15 mice. PAS staining of E15 kidneys from p57+/+ (A) and p57-/- (B) mice shows that all stages of normal glomerulogenesis are present, including maturational glomeruli. Serial sections were stained for BrdU in p57+/+ (C) and p57-/- (D) mice. BrdU is concentrated in the cortical periphery, that is, the location of primitive glomerular structures, and the pattern of immunostaining was indistinguishable in +/+ and -/- mice (magnification $\times 200$).

embryonic mice compared to p57+/+ embryonic mice of the same gestational age (Fig. 3). These results show that p57 is not essential for normal glomerular development.

p57 expression decreased following injury in experimental glomerular diseases

Our results showed that p57 protein was expressed in normal adult mouse glomeruli and normal adult rat glomeruli, and that the localization was specific for podocytes. To determine if p57 protein levels are altered by podocyte injury and if the levels of p57 are associated with changes in proliferation in mature podocytes, we studied two models of experimental glomerular disease.

In the passive Heymann nephritis (PHN) model of experimental membranous nephropathy in the rat which is characterized by very low grade DNA synthesis, but without an increase in podocyte cell number [6], complement-induced injury to rat podocytes was associated with a diffuse and global decrease in the intensity of immunostaining for p57 at days 5 and 10 of PHN compared to normal and control animals (Fig. 4 A, B). There were no obvious differences in p57 staining in polyploid podocytes. Staining for p57 had normalized in podocytes by day 30 of PHN (results not shown).

In the mouse, a model of immune-mediated glomerulonephritis characterized by occasional podocyte prolifer-

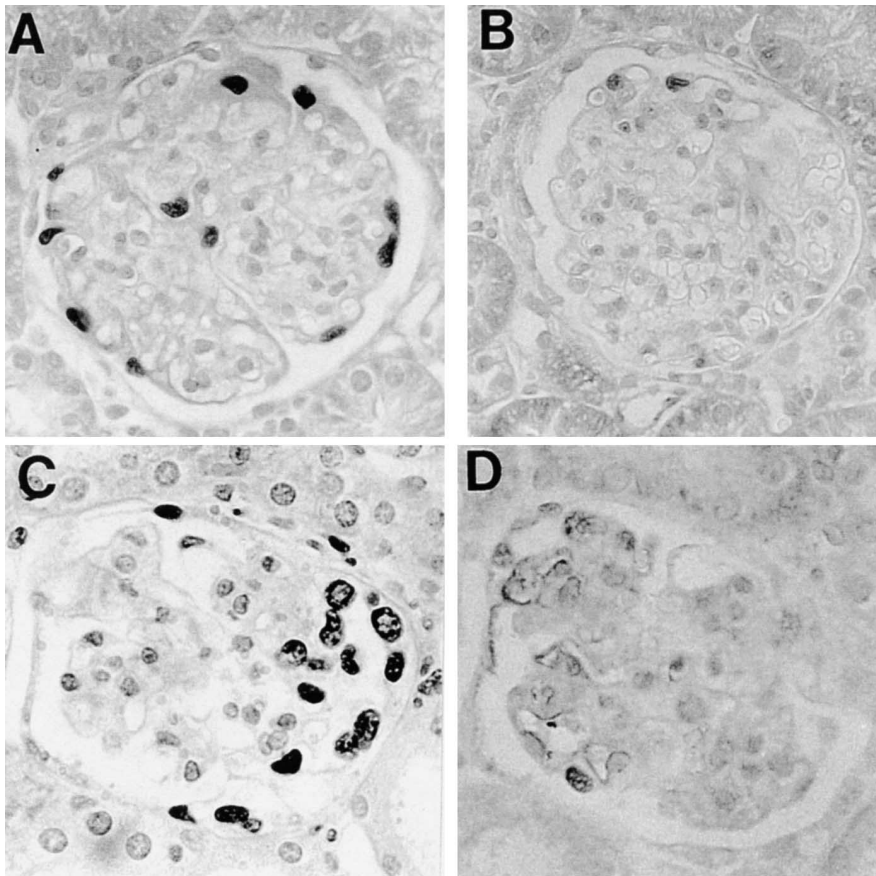


Fig. 4. Indirect immunoperoxidase immunostaining for p57 in experimental glomerular disease. (A) Control rat. There is intense glomerular nuclear staining for p57 in a control rat receiving normal sheep serum, and the distribution is exclusively in podocytes. (B) Day 5 passive Heymann nephritis (PHN). There is a global decrease in p57 staining following C5b-9 injury in PHN. (C and D) Serial sections from murine Anti-GBM glomerulonephritis. (C) Staining for PCNA, a marker of DNA synthesis, is segmentally increased in areas characterized by epithelial cell proliferation. (D) Serial section of the kidney from (C) showing markedly reduced (essentially absent) staining for p57 in the affected glomerulus (magnification $\times 630$).

eration in wild-type mice was studied [20, 34]. In contrast, podocyte proliferation is marked in similarly injured p21 $^{-/-}$ and p27 $^{-/-}$ mice [20, 21]. Diseased mice were sacrificed on day 7, the time of peak proliferation in this model [34]. Our results showed that p57 staining was uniformly absent in mouse podocytes localized to areas of epithelial cell proliferation. Furthermore, serial sections revealed that p57 staining was not detectable in PCNA-positive epithelial cells (Fig. 4 C, D). Moreover, in many glomeruli involved with epithelial proliferation, all murine podocytes appeared to have lost constitutive expression of p57 (Fig. 4 C, D).

Differentiation and quiescence of cultured podocytes was associated with a differential expression of CDK-inhibitors

To determine if the changes observed *in vivo* were reproducible *in vitro*, we studied heat sensitive mouse podocytes in culture. As previously reported, these cells differentiate, arborize and cease to proliferate when grown under certain conditions [37]. Consistent with previous reports [37], analysis of BrdU staining confirmed that under growth permissive conditions, $50 \pm 11\%$ of cells incorporate BrdU after a four-hour pulse. Three days after passage into differentiating conditions, $36 \pm 8\%$ of

cells incorporate BrdU. By five days of culture under differentiating conditions, the rate of DNA synthesis falls to $9 \pm 6\%$ ($P < 0.002$ vs. cells pulsed under growth permissive conditions), which persists out to day 9. All cells grown under permissive and differentiating conditions stained for the Wilms tumor antigen (WT-1), a marker of both developing and mature podocytes *in vivo*. In contrast, only cells grown under differentiating conditions stained for synaptopodin, a marker of mature podocytes, as previously reported (results not shown).

Quantitative real-time PCR was used to determine if the mRNA levels for p21, p27 and p57 were altered upon differentiation of conditionally immortalized mouse podocytes. Figure 5 shows a representative result of the p57 real-time PCR and the standard curve that was generated. Standard curves were also determined for p21, p27 and GAPDH (data not shown) and mRNA expression in each sample was calculated using the standard curve. As shown in Figure 6A, the ratio of p21/GAPDH increased in differentiating podocytes compared to undifferentiated podocytes, and the increase was 15.3-fold in fully differentiated podocytes at day 8. The ratio of p27/GAPDH was only moderately increased (3.8-fold) in differentiating podocytes at day 3 and was 1.6-fold greater in fully differentiated podocytes at day 8 (Fig.

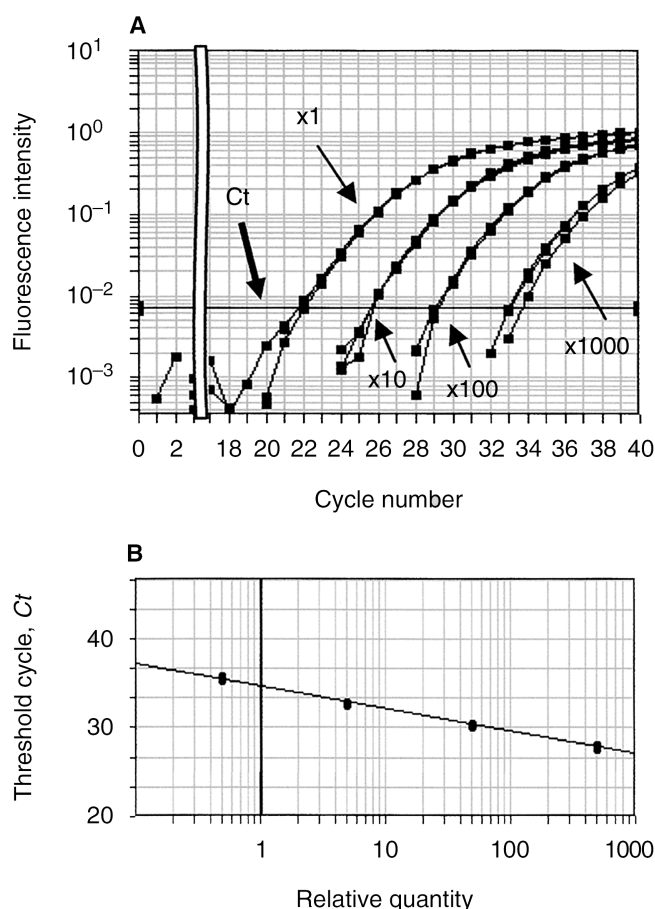


Fig. 5. Quantitative real-time polymerase chain reaction (PCR) for p57mRNA. (A) mRNA was harvested at day 8 growth restrictive conditions. cDNA was then generated by reverse transcription, and the cDNA was diluted 1, 10, 100 and 1000 times for PCR reaction (represented by X1, X10, X100 and X1000 respectively). The PCR product of each dilution at each PCR cycle (X axis) was measured by the intensity of fluorescence (Y axis). Each experiment was performed in triplicate, and the curves at each dilution were superimposed. The results show an exponential increase of fluorescent intensity was observed in certain ranges of PCR cycles. The threshold cycle (Ct) was determined using Sequence Detector™ 1.7. (B) A standard curve was generated for p57 mRNA from the PCR result obtained from each dilution. The relative p57 mRNA quantity of each sample was calculated based on its Ct value using this standard curve. The relative quantity of mRNA is shown on the X axis.

6B). Similar to p21, the ratio of p57/GAPDH was also increased progressively in differentiating podocytes, and was 7.3-fold increased in fully differentiated podocytes at day 8 (Fig. 6C).

Western blot analysis was used to detect the protein expression for p21, p27, and p57 from cells grown under growth permissive and differentiating conditions (Fig. 7). p21 was undetectable under growth permissive conditions. In contrast, p21 expression increased gradually in differentiated podocytes and was maximally expressed in fully differentiated podocytes. p27 was expressed in low abundance under growth permissive conditions and

early following the passage into differentiating conditions. However, there was an increase in p27 levels in differentiated podocytes on day 8.

In contrast to the Cip/Kip CDK-inhibitors p21 and p27, protein levels for p57 were barely detected in cultured podocytes by Western blot analysis (Fig. 7). This was not a false negative, because p57 protein could be detected easily in the positive control protein, and this was verified by antibody absorption studies. Although we typically use 10 to 20 μ g of total podocyte protein lysate for Western analysis, we were still unable to detect significant amounts of p57 protein using 100 μ g of podocyte protein lysate. These findings were reproducible in duplicate blots performed on protein lysates obtained from four separate experiments.

Differentiation of heat-sensitive mouse podocytes was associated with a decrease in expression of cyclins and CDKs

Given the possible role of negative cell cycle regulators (CDK-inhibitors) to modulate the terminal differentiation of conditionally immortalized mouse podocytes, we sought to clarify whether positive cell cycle regulators also could regulate differentiation and proliferation. Therefore, Western blot analyses for cyclin A, cyclin B, and CDK2 was performed on cell lysates obtained at day 3 following passage in permissive conditions and at days 3 and 8 following passage into differentiating conditions (Fig. 8).

Our results showed that cyclin A, cyclin B, and CDK2 protein were abundant under growth permissive conditions. In contrast, after passage into differentiating conditions, cyclin A expression was markedly decreased and cyclin B levels were undetectable. Cyclin D2 was detectable in low abundance under growth permissive conditions. In contrast, D2 levels increased in differentiated podocytes, with peak expression demonstrable by the fully differentiated phenotype on day 8. CDK2 protein was present in proliferating cells, but the change to a differentiated phenotype coincided with a decrease in CDK2 levels.

DISCUSSION

The mature podocyte has a limited proliferative capacity, and the failure of injured podocytes to repopulate a denuded glomerular basement membrane (GBM) may underlie the formation of tuft adhesions and progressive glomerulosclerosis in certain diseases [1, 39, 40]. To delineate the mechanisms underlying these events further, the current study focused on the CDK-inhibitor p57 because it is constitutively expressed in the normal podocyte in mice, rats and man [30]. Our results showed that despite the de novo increase in p57 protein expression during glomerulogenesis, p57^{-/-} mice have normal glo-

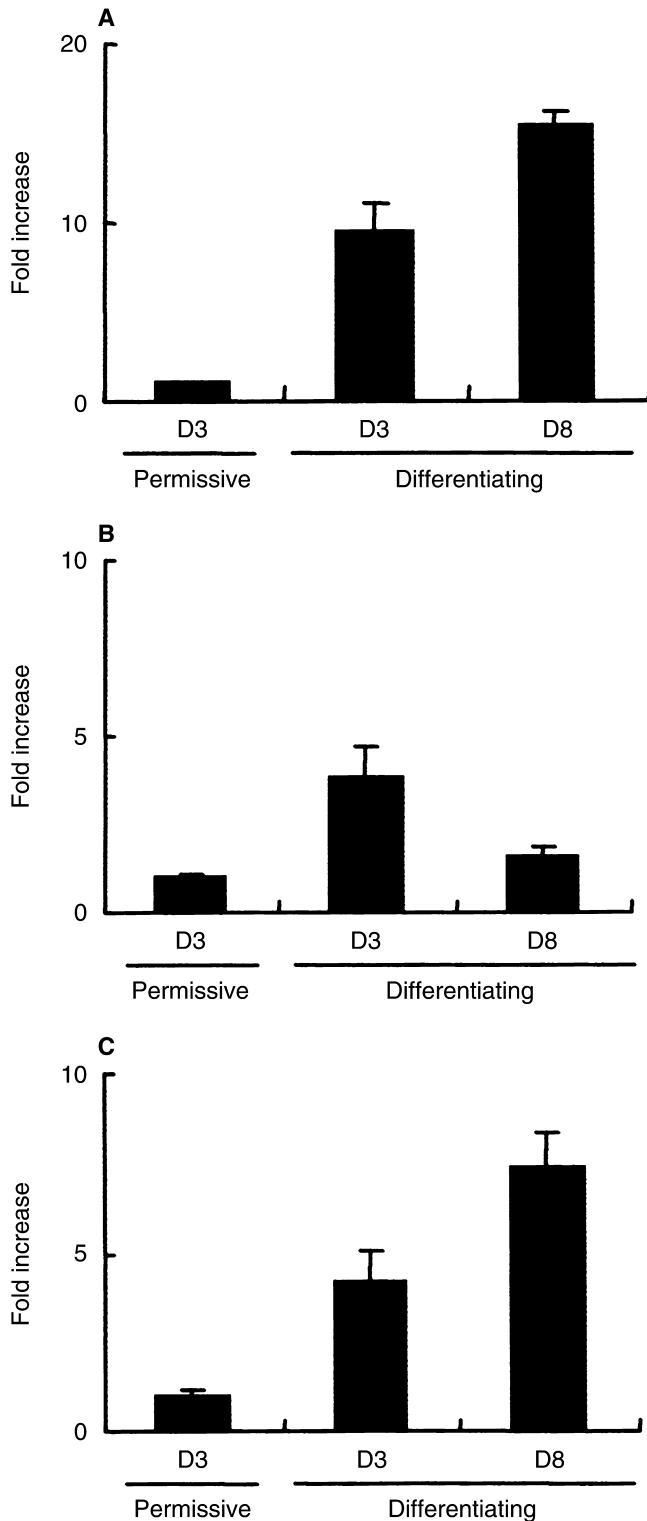


Fig. 6. p21, p27 and p57 mRNA expression in cultured podocytes measured by real-time PCR. (A) The ratio of p21/GAPDH mRNA is strikingly increased at day 3 (D3) and day 8 (D8) of growth restrictive conditions (differentiating) compared to growth permissive podocytes at day 3 (D3). (B) The ratio of p27/GAPDH mRNA is moderately increased at day 3 and slightly increased at day 8 of growth restrictive conditions (differentiating) compared to growth permissive conditions at day 3 (D3). (C) The ratio of p57/GAPDH mRNA is moderately increased at day 3 and markedly increased at day 8 in growth restrictive

merular morphology. Furthermore, we showed that p57 mRNA levels also increased in podocytes undergoing arborization and differentiation in vitro. Finally, immune-mediated injury to mature podocytes in experimental glomerular disease was associated with a decrease in p57 levels, but the loss of p57 alone was not sufficient to cause proliferation.

Our previous studies have focused on the role of the Cip/Kip CDK-inhibitors p21 and p27 in podocytes under normal and diseased states [19–21]. However, little is known about the role of p57 (the third member of the Cip/Kip family of CDK-inhibitors) in podocytes. Accordingly, we began this study by extending the work of Nagata and colleagues [31] and our previous studies [41] to determine if p57 was essential for podocyte development. Our data showed that the serial expression of p57 was most readily detected at the capillary loop stage, and that mature podocytes constitutively expressed p57. Interestingly, the loss of podocyte proliferative markers during development and the transition to the mature phenotype coincided with the onset of p57 protein expression. This descriptive data suggested that p57 is essential for normal glomerulogenesis. However, the first major finding in the current study was that p57^{−/−} mice have normal glomerular morphology and, contrary to our expectation, p57^{−/−} mice did not exhibit an increase in glomerular DNA synthesis or cell number. Unfortunately, because the majority of these mice die at birth from presumed non-renal causes [32, 42], we did not have adequate numbers to assess glomerular development postnatally. Taken together, our data suggest that although p57 expression coincides with the acquisition of a mature phenotype, this CDK-inhibitor alone is not essential for podocyte development, whereas it is for other organs comprising the gastrointestinal and skeletal systems [32, 42, 43]. Moreover, our results show that within the kidney, there is a differential role for p57 in development, as Zhang has previously shown that p57 null mice develop medullary dysplasia [32]. Interestingly, although p21 and p27 also are expressed during glomerular development, p21 null [20] and p27 null mice [21] both have a normal glomerular architecture. Thus, the current and previous studies support the notion that individual members of the Cip/Kip family of CDK-inhibitors alone are not required for podocyte maturation. We await the histology of double CDK-inhibitor null mice.

In order to determine the potential role of specific CDK-inhibitors in podocyte differentiation in vitro and to examine if the in vivo results described earlier in this article also occurred in vitro, we studied a conditionally

conditions (differentiating) compared to permissive conditions. Real-time PCR was performed in triplicate.

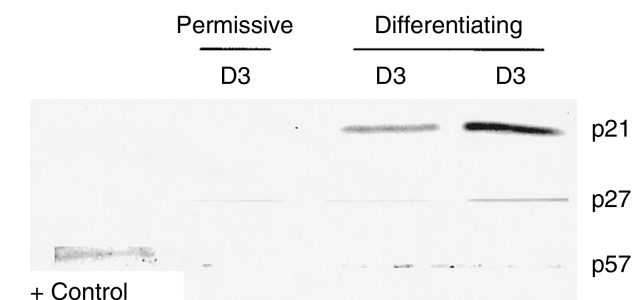


Fig. 7. Western blot analysis for CDK-inhibitors in cultured podocytes. Expression of p21 and p27 increase upon podocyte differentiation (20 μ g total protein in each lane). The protein levels of p57 were barely detected in differentiated podocytes (100 μ g total protein in each lane). A positive (+) control using the same antibody is shown, indicating that the inability to detect a stronger band for p57 was not a false negative.

immortalized mouse podocyte cell line that has been previously reported to show many of the characteristics of podocytes *in vivo* [37]. The second major finding in this study was that the change in phenotype from a growth permissive one to an arborized and growth restrictive phenotype was associated with increases in the mRNA levels for p21, p27 and p57. This was accompanied by an increase in protein levels for p21 and p27. Our results also showed that, in contrast to an increase in cyclin D2, the switch to a mature phenotype *in vitro* was associated with a decrease in cyclins A and B1, and CDK2. These results show that podocyte differentiation *in vitro* involves a complex interplay of cyclin-CDKs and specific CDK-inhibitors. Moreover, these results show that p57 may not play a significant role in podocyte differentiation *in vitro*, a result similar to the *in vivo* studies. We can only speculate why p57 mRNA but not protein levels did not increase upon differentiation *in vitro*. One possibility is increased p57 degradation. However, these results should not be interpreted to imply that this is an invalid cell culture model, because previous studies have shown that heat-sensitive mouse podocytes have many features consistent with podocytes *in vivo*, and thus is a very useful model to further our understanding of podocyte biology.

We [16] and others [31] have recently shown that p57 is constitutively expressed in mature human podocytes. To determine if p57 levels were altered by injury and if this was associated with changes in the podocyte cell cycle, we studied two models of glomerular disease characterized by podocyte injury. The third major finding in this study was that injury to podocytes in experimental passive Heymann nephritis (PHN) and in anti-glomerular antibody induced glomerulonephritis was associated with a decrease in p57 protein expression. However, our results showed that despite a decrease in p57 expression, the capacity of podocytes to re-engage the cell cycle and

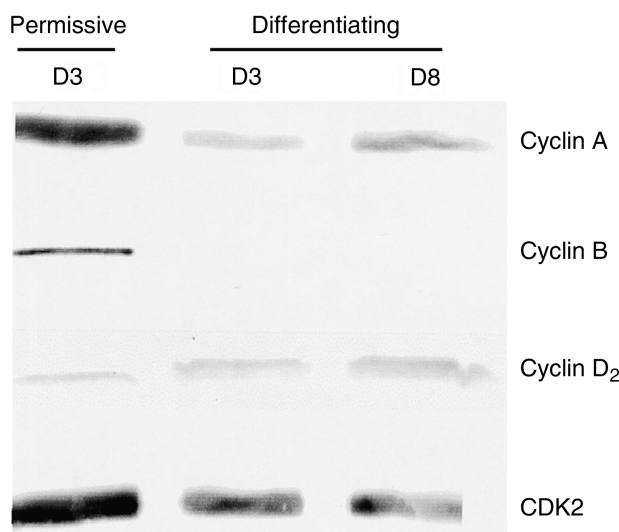


Fig. 8. Western blot analysis of positive cell cycle regulatory proteins in differentiating mouse podocytes *in vitro*. Western blots were performed on protein lysates obtained 3 days following passage in growth permissive conditions, and days 3 and 8 following passage into differentiating conditions. Cyclin A, cyclin B, and CDK2 are all strongly expressed under permissive conditions and the protein levels were markedly reduced after passage into differentiating conditions. In contrast, Cyclin D2 expression increased after passage into differentiating conditions.

proliferate was determined by the balance of Cip/Kip CDK-inhibitors, rather than p57 alone. Thus, in PHN rats, the transient and early decrease in p57 expression was not associated with any marked increase in podocyte proliferation. We have previously shown that the apparent inability of podocytes to proliferate in PHN rats was associated with an increase in p21 and p27 levels, and that decreasing p21 levels by administering the mitogen basic fibroblast growth factor (bFGF) was associated with increased DNA synthesis [19]. In contrast, the increase in podocyte proliferation in p21^{-/-} [20] and p27^{-/-} [21] mice with glomerulonephritis induced with an anti-glomerular antibody was associated with a marked decrease in p57 expression (unpublished data).

We recently showed that p57 expression was not altered in human membranous nephropathy [30]. Our current study shows that there was an early and transient decrease in p57 in experimental membranous nephropathy (PHN model), but the levels normalized within 30 days after injury. Thus, the duration of disease may be a plausible explanation accounting for the differences in human and experimental membranous nephropathy. Injecting mice with the anti-glomerular antibody described is associated with an increase in podocyte proliferation that results in layers of podocytes in association with collapse of the glomerular tuft. We recently showed that the ability of podocytes to proliferate in human glomerular disease correlates closely with p57 levels. In cellular and collapsing focal segmental glomerulonephri-

tis, p57 levels decreased in areas of proliferation [30]. These results are consistent with the current study where we showed that podocyte proliferation in a model of anti-glomerular antibody induced glomerulonephritis correlated with a decrease in p57 levels. Taken together, we propose that, although injury to podocytes causes a decrease in p57 expression, this alone is not sufficient to cause proliferation, and that the balance of p21, p27 and p57 levels determines the proliferative potential of podocytes in response to injury. We also suggest that examining p57 protein expression may be a useful marker of podocyte injury. Finally, we have not determined the mechanisms whereby p57 levels decrease in glomerular disease.

In summary, this study shows that the glomerular expression of the CDK-inhibitor p57 localizes specifically to the mature podocyte, and first appears at the capillary loop stage of glomerulogenesis. However, we show that p57 is not essential for normal glomerular development in vivo, and that p57 protein levels do not change upon switching from a growth permissive to a differentiated state in vitro. Our results also show that injury to podocytes in experimental glomerular disease is associated with a decrease in p57 expression, but that the proliferative capacity of podocytes is determined by the balance of all three members of the Cip/Kip family of CDK-inhibitors.

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Reprint requests to Stuart J. Shankland, M.D., Division of Nephrology, University of Washington School of Medicine, Box 356521, Seattle, Washington 98195, USA.
E-mail: stuartjs@u.washington.edu

APPENDIX

Abbreviations used in this article are: BrdU, 5-bromo-2'-deoxyuridine; CDK, cyclin dependent kinase; CKI, cyclin kinase inhibitors; E, embryonic; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFR, glomerular filtration rate; HSMP, heat-sensitive mouse podocyte; p21, p21^{CIP1}, WAF1, SDI1; p27, p27^{KIP1}; p57, p57^{KIP2}; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PHN, passive Heymann nephritis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT-1, Wilms tumor-1.

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